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Embryonic stem cell miRNAs and their roles in Development and Disease

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Abstract

MicroRNAs have emerged as important modulators of gene expression. Both during development and disease, regulation by miRNAs controls the choice between self-renewal and differentiation, survival and apoptosis and dictates how cells respond to external stimuli. In mouse pluripotent embryonic stem cells, a surprisingly small set of miRNAs, encoded by four polycistronic genes is at the center of such decisions. miR-290~295, miR-302~367, miR-17~92 and miR-106b~25 encode for miRNAs with highly related sequences that seem to control largely overlapping gene sets. Recent studies have highlighted the importance of these miRNAs in the maintenance of 'stemness' and regulation of normal development and have linked the deregulation of their expression to a variety of human diseases.

Introduction

MicroRNAs (miRNAs) were first described almost 20 years ago by the groups of Ambros and Ruvkun. In two seminal papers, they reported the identification of a small RNA able to control developmental timing in *C. elegans* through post-transcriptional regulation of a protein-coding gene [1, 2]. Since then, the catalog of miRNAs and processes controlled by them has grown beyond the expectation of most. We now know that gene regulation by miRNAs is a conserved mechanism across animals and plants. In humans alone, over 1000 miRNAs have been identified, placing them amongst the largest classes of gene regulatory molecules in the genome [3]. As the number of described miRNAs steadily increases, so does that of their putative targets, with the latest predictions pointing to over a third of all human genes being under such control [4, 5]. The ubiquitous nature of miRNAs has changed the way we look at the regulatory networks dictating cellular behavior during a variety of biological processes: we simply cannot fully understand them without accounting for miRNA action.

Despite the well-recognized role of miRNAs in controlling gene expression, relatively little is known about their physiological functions and the mechanisms by which they drive disease. MicroRNAs have been proposed to confer robustness to cell fate decisions [6–8], ensuring fast and accurate control over protein output and modulating the responsiveness of cells to external signals [9]. This is particularly important during embryogenesis, when

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expression programs have to be changed within very short developmental windows. In the early blastocyst-stage embryo for example, a small population of pluripotent epiblast cells is responsible for giving rise to all cell types of the adult organism. The switch between pluripotency and the onset of differentiation requires massive changes in gene expression involving silencing of genes controlling the undifferentiated state and activation of the appropriate differentiation programs. These changes are achieved through a complex interplay of signaling pathways, transcription factors, chromatin remodeling proteins and miRNAs (reviewed in [10]).

The mechanisms controlling pluripotency and differentiation during early development have been best studied in embryonic stem cells (ESCs), which derive from the epiblast of a blastocyst-stage embryo. These cells can proliferate indefinitely *in vitro* and —like the cells they originate from— are poised to differentiate when exposed to proper signals. Like any other kind of specialized cell, ESCs express a specific set of miRNAs that are an intricate part of the regulatory program that controls their phenotype. Surprisingly, in the case of mouse ESCs, the largest portion of miRNAs is encoded by four polycistronic loci harboring miRNAs with highly related seed-sequences: miR-290~295, miR-302~367, miR-17~92 and its paralog cluster miR-106b~25. Members of these clusters have been implicated in the maintenance and establishment of pluripotency as well as regulation of differentiation and tissue formation during embryo development. Expression of these miRNAs is often found deregulated in a variety of human cancers, placing them not only at the center of developmental decisions but also as key players in the pathogenesis of human malignancies. In this review, we present an overview of our current knowledge about these four clusters of miRNAs, we discuss the processes they control during development, and we suggest possible mechanisms through which their deregulation may lead to diseases.

The miRNA biogenesis pathway

The molecular mechanisms controlling miRNA biogenesis have been extensively reviewed in the past [11]. MicroRNAs, typically 22 nucleotide-long, repress gene expression by base-pairing to complementary regions on the 3'UTR of their targets and preventing protein accumulation through mRNA destabilization and/or translation inhibition [5]. They are transcribed as long primary transcripts (pri-miRNAs) [12] and need to undergo two consecutive processing steps to give rise to mature miRNAs. The first maturation step occurs in the nucleus where pri-miRNAs are trimmed into shorter hairpin-shaped precursors (pre-miRNAs). For canonical miRNAs, this process is catalyzed by Drosha together with its co-factor Dgcr8 [13–15], whereas mirtrons—located in introns of protein coding genes—rely on the splicing machinery [16, 17]. The precursor miRNAs are exported to the cytoplasm by Exportin-5 [18] where they are further processed by Dicer into ~22-nucleotide long miRNAs [19, 20]. These mature molecules are recognized and loaded into the RNA-induced silencing complex (RISC), where one of the miRNA strands is used as a guide to target transcripts. Target recognition depends heavily on the miRNA seed-sequence — a stretch of 6 nucleotides spanning from position 2 through 7— as a determinant of specificity [21–23].

Even though the level of repression exerted by miRNAs is relatively modest, leading to only minor adjustments in protein output [24, 25], their regulatory action is essential for a variety of biological processes, as shown by deletion of proteins involved in their maturation pathway. These processes include differentiation, proliferation and self-renewal [26–29], and their deregulation is a key event in cellular transformation and tumor progression [30].

miRNAs in ESC function

During embryogenesis, the balance between pluripotency and differentiation needs to be tightly controlled both in time and space. Because the early embryo provides limited biological material to probe for, most of the studies regarding regulation of pluripotency and early lineage decisions have been done using ESCs as a surrogate for the epiblast population. These studies, have recently highlighted the importance of miRNAs in the regulatory network that controls cell fate and have placed miRNAs amongst the key players of embryogenesis.

miRNAs are required for ESC function and early mammalian development

The first evidence that miRNAs play essential roles in the maintenance of the ESC phenotype and are required for early mouse development came from the analysis of mutant cells carrying the targeted deletion of the genes encoding for Dicer and Dgcr8. It is worthwhile noting however, that while Dgcr8 has been implicated only in the miRNA pathway, Dicer also seems to be involved in the maturation of endogenous siRNAs [31], and therefore, extrapolations of miRNA function based on the phenotype of its knockout need to be done with caution.

Dicer is essential for vertebrate development and its deletion leads to early embryonic lethality [32, 33]. In agreement with this finding, Dicer-null mouse ESCs—though viable and morphologically normal—differentiate aberrantly and fail to contribute to the generation of chimeric mice when re-introduced into blastocysts. Furthermore, they do not form teratomas when injected subcutaneously into nude mice and fail to up-regulate differentiation markers during embryoid body formation *in vitro*. In addition, although they express pluripotency markers at levels comparable to those of wild-type cells, Dicer-null ESCs are unable to fully silence them upon induction of differentiation [26]. In the absence of Dicer, ESCs also proliferate slower and show an altered cell cycle profile compared to their wild-type counterparts, with accumulation of cells in G1 [34]. Even though theoretically these defects can be attributed to lack of both miRNAs and endo-siRNAs, miRNAs seem to be the sole substrate for Dicer in ESCs [35], and therefore are likely the cause of the defects observed in its absence.

Like mutants for Dicer, mice lacking Dgcr8 die around gastrulation and Dgcr8-null ESCs, although morphologically normal, have proliferation defects with accumulation of cells at G1. Dgcr8^{-/-} ESCs also differentiate abnormally, though this defect is not as severe as the one observed in the absence of Dicer: they are able to up-regulate markers for all germ layers albeit slower and to a lesser degree than wild-type cells. Like Dicer^{-/-} cells however, they are unable to silence pluripotency markers such as Oct4, Rex1, Sox2 or Nanog [29].

Together, these studies indicate that an intact miRNA pathway is absolutely required for viable embryo development, and that its inactivation results in defects in ESC proliferation and differentiation.

ESCs express a specific set of miRNAs

The studies described above place the miRNA pathway among the regulatory mechanisms controlling ESC function and, by extension, early embryonic development, but they do not address which miRNAs contribute to the defects observed. As a first attempt to tackle this issue, several groups have cloned and sequenced small RNAs from both mouse (mESC) and human embryonic stem cells (hESC) and found that they express a unique set of microRNAs [35–38].

At the top of the list of miRNAs cloned from mouse ESCs are those belonging to the miR-290~295 cluster, which encodes for seven distinct miRNAs, six of which share the same seed-sequence ('AAGUGC') ([36, 39], see Table 1). The remaining top positions are occupied by members of the miR-17~92, miR-302~367 and miR-106b~25 clusters among others. Interestingly, these three polycistronic genes also contain miRNAs with the 'AAGUGC' seed or the variation of it, 'AAAGUG', suggesting that it might be an important feature of the ESC regulatory network. In line with a role for these miRNAs in ESC function, the 3'UTRs of transcripts up-regulated in Dicer-null ESCs are enriched for the 'GCACUUU' and 'AGCACUU' motifs, complementary to these seed sequences [40], and a recent study, queering mRNA-miRNA interactions in mouse ESCs using the newly developed HITS-CLIP technique [41], has found that members of the miR-290~295 and the miR-302~367 clusters represent the largest fraction of miRNAs that co-precipitate with Argonaute2 [42].

As in the mouse, human embryonic stem cells are also enriched in miRNAs from clusters expressing the 'AAGUGC' and 'AAAGUG' seeds, which include members of the miR-302~367 cluster—the most abundant miRNA cluster in hESC—members of the miR-371~373 cluster, which belongs to the miR-290 family of miRNAs [39], as well as members of the miR-17~92 cluster [37, 38]. This suggests that the mechanisms by which these miRNAs control ESC function are conserved between human and mouse.

Despite the prevalence of the 'AAGUGC/AAAGUG' seed among these four clusters, all of them also express miRNAs with unrelated sequences (Table 1). This polycistronic organization, where a single primary transcript yields distinct pre-miRNAs may have been favored because it allows the co-regulation of miRNAs that need to be co-expressed.

ESC-miRNAs are part of the regulatory circuitry that controls pluripotency

Since they first were isolated [43, 44], ESCs — and the mechanisms that control their pluripotency and differentiation — have been the subject of intense research. In the past years, Oct4, Sox2 and Nanog, whose expression is essential for the maintenance of pluripotency in the mouse epiblast as well as in ESCs [45–49], have emerged as the core pluripotency factors [10]. Indeed, their forced expression in combinations with c-Myc, Klf4, Lin28 and Tcf3 has been shown to reprogram both murine and human terminally differentiated cells into pluripotent (ESC-like) cells, capable of giving rise to all three germ layers when induced to re-differentiate [50–53]. Genome-wide mapping of binding sites for these factors has helped elucidate the mechanisms by which they control pluripotency and self-renewal [54–57]. Oct4, Sox2 and Nanog co-occupy the promoters of hundreds of genes, often together with the other pluripotency factors. Binding to the promoters leads to activation of genes involved in the maintenance of the ESC program — which includes activation of their own expression — as well as repression of genes involved in lineage differentiation, frequently through co-occupancy by Polycomb group proteins [57].

MicroRNAs, like protein-coding genes, are also direct transcriptional targets of the pluripotency factors (Figure 1). More specifically, the promoters of miR-290~295 and miR-302~367 are bound and activated by Oct4, Sox2, Nanog and Tcf3 in mouse ES cells [55], while miR-17~92 has been shown to be a direct transcriptional target of c-Myc [58]. Interestingly, the function of c-Myc in ESCs seems to differ from that of the other factors. Oct4, Sox2 and Klf4 often co-occupy promoters, but these have only a limited overlap with those bound by c-Myc [54]. A subsequent study has shown that c-Myc enforces pluripotency only in the initial steps of reprogramming by down-regulating fibroblast-specific genes and facilitating the binding of the remaining factors [59]. Reprogramming of differentiated cells without the use of ectopic c-Myc has been reported, though with a

markedly reduced efficiency [60] and while disruption of its endogenous locus does not by itself affect ESC function [61], deletion of c-Myc in ESCs along with its family member N-myc —another transcriptional activator of miR-17~92 [62, 63]— triggers early lineage commitment and cell cycle arrest, demonstrating that their combined activity is required for the maintenance of the ESC phenotype [64, 65].

Like the pluripotency factors themselves, the AAGUGC/AAAGUG expressing clusters have also been implicated in the induction of pluripotency. Ectopic expression of members of miR-106b~25 and miR-302~367 enhances reprogramming by Oct4/Sox2/Klf4/c-Myc [66, 67] while expression of miR-290~295 members enhances reprogramming by Oct4/Sox2/Klf4 but not Oct4/Sox2/Klf4/c-Myc [68]. Surprisingly, a recent work has shown that miR-302~367 alone, in the presence of the HDAC inhibitor VAP, is sufficient to dedifferentiate fibroblasts into induced pluripotent cells [69]. This reprogramming protocol not only bypasses the requirement for the use of the pluripotency TFs but also leads to a 100-fold increase in iPS colony formation efficiency when compared to that achieved by the Oct4/Sox2/Klf4/c-Myc cocktail in the presence of the same drug [69]. Reprogramming by miR-302~367 requires the presence of both the miR-302 and the miR-367 seeds, indicating that the miRNAs encoded by this cluster functionally cooperate during the acquisition of pluripotency.

The specific mechanisms by which the miR-302~367 miRNA cluster can so efficiently promote reprogramming remain to be elucidated, although some interesting hypotheses have been put forward. Among the genes regulated by members miR-302~367 are multiple targets that can influence the dedifferentiation of somatic cells (Figure 1). These include regulators of the cell cycle (like p21, Lats2 and Rbl2; see below), epigenetic regulators (such as Mecp2, Smarcc2) and genes involved in epithelial-to-mesenchymal transition (EMT; such as RhoC, and TGFβRII) [66, 70]. Indeed, repression of p21, Rbl2, RhoC and TGFβRII has been shown to increase the efficiency of reprogramming [66, 67, 71], thus suggesting a possible mechanism for miR-302~367 action. Members of the miR-17~92 and miR-106b~25 clusters have also been shown to facilitate reprogramming by down-regulating p21^{Cip1} and TGFβRII [67], and p21^{Cip1}, Lats2, Rbl2 and TGFβRII have all been shown to be *bone fide* targets not only of these two clusters but also of miR-290~295 [70, 72–75], suggesting that they all help reprogramming by targeting largely overlapping gene sets.

The function of the four clusters in the regulation of pluripotency is opposed by the let-7 family of miRNAs [76], of which several members exist in mouse and human genomes [77]. Let-7 miRNAs are expressed in a broad range of differentiated tissues [78] but their maturation is blocked in ESCs by LIN28 [79, 80], a conserved RNA binding protein that selectively binds to let-7 precursors [81] thereby preventing their processing [82–84], and also one of the few proteins known to induce pluripotency ([51] and see above). The mechanism by which LIN-28 enforces pluripotency may rely on its ability to inhibit let-7 function. Indeed, inhibition of this family of miRNAs by anti-sense molecules was shown to increase the rate of reprogramming by Oct4/Sox2/Klf4 by four-fold, whereas forced expression of mature let-7 RNAs in Dgcr8^{-/-} mESCs, led to silencing of the self-renewal program [76]. Interestingly, co-transfection of mature let-7 and miR-294 miRNAs in Dgcr8^{-/-} mESCs or transfection of let-7 alone in wild-type mESCs, abolishes the ability of these miRNAs to repress the stem-cell fate, thus suggesting that the two sets of miRNAs control opposing expression programs to fine-tune the balance between pluripotency and differentiation [76].

More recently, the miR-34 family of miRNAs has also been implicated in the regulation of pluripotency [85]. Reprogramming of differentiated cells is a process that occurs at low efficiency, due to existence of molecular barriers —most notably the p53 pathway— that

prevent improper acquisition of self-renewal [71, 86–88]. MicroRNAs from the miR-34 family show a p53-dependent activation during the induction of pluripotency and suppress reprogramming at least in part through repression of pluripotency genes such as Nanog, Sox2 and N-myc [85]. In the absence of miR-34 microRNAs, reprogramming by Oct4/Sox2/klf4 or Oct4/Sox2/Klf4/c-Myc increases by more than four-fold, and co-deletion of miR-34a and p21—another transcriptional target of p53—indicate that the two cooperatively mediate repression of reprogramming downstream of p53 [85].

ESC-miRNAs regulate the Cell Cycle

In somatic cells, commitment to proliferate requires overcoming the G1-to-S restriction (R) point, after which progression through the cell cycle becomes independent of external growth signals [89, 90]. Control over the G to S transition, which relies largely on regulation of the retinoblastoma protein (pRB) activity, is critical for the maintenance of tissue homeostasis and for correct differentiation. In its active state, pRB sequesters E2F transcription factors and prevents them from activating the expression of target genes. In response to mitogenic signals however, pRB is sequentially phosphorylated leading to its inactivation and consequently, allowing progression through the cell cycle. Phosphorylation of pRB occurs in two steps. Cyclin D, whose expression is induced by the external mitogenic signals, complexes with CDK4 and CDK6 and leading to phosphorylation of pRB, partial release of the E2F factors and activation of their downstream targets Cyclin E and Cdc25A. Cdc25A then removes phosphate groups from CDK2, allowing it to complex with Cyclin E and further phosphorylate pRB to achieve full release of E2F and progression into the S phase [91, 92]. In a parallel branch of the network, mitogenic signals induce c-Myc expression [93], which independently activates the Cyclin E and Cdc25A promoters [94]. The activity of CDK proteins is regulated by inhibitors that are activated in response to growth arrest signals such as contact inhibition, senescence and differentiation: members of the INK4 family (p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}) bind to and inhibit CDK4 and CDK6, while members of the Cip/Kip family (p21^{Cip1}, p27^{Kip1} and p57^{Kip2}) inhibit the activity of CDK2 [95].

In striking contrast to somatic cells, ESCs do not have an R point, allowing them to progress rapidly through G1 even in the absence of growth signals. ESCs display constantly high levels of active Cyclin E/CDK2 complexes leading to a constitutively hyperphosphorylated pRB and transcription of E2F targets. Inactivation of pRB seems to be independent of Cyclin D complexes, which are present in ESCs at very low levels [96]. Also in contrast to somatic cells, ESCs do not express CDK inhibitors [96–98], which helps ensure high levels of Cyclin E/CDK2 complexes, the rate limiting factors in ESC proliferation [96]. The recent discovery that miRNA-deficient ESCs accumulate at G1 indicates that they are also part of the network that ensures a rapid G1-to-S transition [26, 29]. In fact, Dgcr8 null cells display abnormally high levels of cell cycle inhibitors such as p21^{Cip1} and Lats2—two regulators of the Cyclin E/CDK2 complex—and Rbl2, a member of the pRB family. This suggests that one of the functions of miRNAs in ESCs is to keep the levels of these inhibitors in check. Indeed, the transcripts of p21^{Cip1}, Lats2 and Rbl2 are direct targets of the ‘AAGUGC/AAAGUG’-seed miRNAs expressed by the miR-290~295, miR-302~367 and miR-17~92 clusters (Figure 2), and the re-introduction of these miRNAs in Dgcr8^{-/-} ESCs rescues not only the expression levels of the inhibitors, but also the proliferation defects of the cells [70]. miR-92, a miRNA of the miR-17~92 cluster expressing a distinct seed has also been implicated in an analogous regulation in human ESCs, by targeting p57^{Kip2} through a site that is predicted to be conserved between humans and mice [99]. p57^{Kip2} is likely to be also a target of miR-367 and miR-25, encoded by the miR-302~367 and miR-106b~25 clusters respectively, since they have identical seed sequences to miR-92 (see table 1).

G1 is the thought to be a window of opportunity for lineage commitment, during which cells are sensitized to differentiation signals [100]. The relatively short G1 phase in ESC cells, maybe a way of safeguarding them from improper differentiation. Indeed, lengthening the duration of G1 in embryonic as well as tissue stem cells has been shown to enable them to differentiate, while shortening this phase prevents them to do so [89, 101–105]. As regulators of G1-to-S transition in ESCs, miRNAs are likely to influence differentiation events during mammalian development, much like other prominent regulators of the cell cycle [106, 107].

ESC-miRNAs regulate repression of Oct4 during differentiation

Apart from a deregulated cell cycle, ESCs without a functional miRNA pathway also display defects in differentiation, with one of the major consequences being the inability to silence pluripotency markers. Several miRNAs are up-regulated during differentiation and show tissue-specific expression in both embryos and adult mice, suggesting that they are involved in lineage decisions (reviewed in [108]). Absence of these miRNAs in *Dicer*^{−/−} and *Dgcr8*^{−/−} mESCs may explain in part their aberrant differentiation and the inability of null embryos to survive gastrulation. However, improper inactivation of pluripotency markers, specifically of Oct4, seems unexpectedly to also result from the absence of miR-290~295, a cluster typically associated with the pluripotent state [40, 109].

Initial silencing of Oct4 during differentiation is achieved by repressors proteins, which bind to its promoter and cause a transient transcriptional repression. Stable and irreversible silencing of this gene however, requires promoter methylation, which is catalyzed by the Dnmt3a and Dnmt3b *de novo* methyltransferases ([110, 111]; Figure 1). *Dicer*-null ESC cells have abnormally low levels of both these enzymes, which result in a global loss of DNA methylation [109] and inability to fully methylate and silence Oct4 [40, 109]. Although repression of Oct4 in knockout cells is initially comparable to wild-type, it is lost in mutant cells at later stages of differentiation, a defect that can be rescued by overexpressing Dnmt3a and Dnmt3b [40, 109]. The low Dnmt3 levels in *Dicer*^{−/−} have been traced back to Rbl2, a known repressor of Dnmts that is upregulated in mutant cells due to lack of repression by miR-290~295 and related clusters (see above; Figure 1 and [112]). Indeed, both knockdown of Rbl2 and transfection of miR-290~295 members can restore the levels of the two enzymes and the methylation of the Oct4 promoter during mESC differentiation. Because Rbl2 is also a direct target of the miRNAs encoded by the remaining ESC-clusters it seems likely that they too are indirectly responsible for silencing of Oct4—and perhaps additional pluripotency factors—during differentiation.

ESC-miRNAs in embryonic development

We have discussed above the role of miRNAs in the mechanisms that control ESC self-renewal, which likely mirror the events that take place in the epiblast of the early mouse blastocyst. In these cell populations, members of the miR-290~295, miR-302~367, miR-17~92 and miR-106b~25 clusters seem to play redundant roles due to their similarity and co-expression. Indeed, knockout mice have been reported for three out the four clusters with no report indicating defects at the level of embryonic stem cell function or pre-implantation development [113, 114]. The roles of the clusters seem to diverge during later stages of development, possibly due to expression in distinct tissues. The primary transcript of miR-290~295 is first detected in 4–8 cell embryos and its expression starts decreasing after embryonic day (E) 6.5. Later in development, miR-290~295 expression is restricted to the germ cells in the gonads, where it is detected up to E13.5 in females and E14.5 in males [114]. Unlike miR-290~295, the transcripts of miR-17~92 and miR-106b~25 are detectable in most tissues even in adult animals, with largely overlapping expression patterns [113].

The expression of miR-302~367 has not been addressed in detail, but seems to reach its peak during early embryogenesis, before germ layers are established, and rapidly decrease with differentiation [78, 115].

miR-17~92 and miR-106b~25

miR-17~92 is essential for mouse development, and its deletion leads to perinatal death due to a variety of developmental defects. Mice that lack this miRNA cluster are significantly smaller than their wild-type littermates and display lung hypoplasia, cardiac defects, a block in B cell development as well as abnormalities in skeletal mineralization and patterning [113, 116]. miR-17~92 and miR-106b~25 encode common seed sequences (see Table 1) and seem to be co-expressed in a variety of different tissues, suggesting that they are involved in the regulation of the same biological processes. Indeed, although deletion of miR-106b~25 by itself does not have any deleterious effect, mice that carry targeted deletions of both clusters die before E15.5 and show more severe phenotypes, suggesting that both genes functionally cooperate.

The defects observed in the absence of miR-17~92 and miR-106b~25 have been attributed in part to increased apoptosis, which is widespread in double knockout mutants. In miR-17~92 null mice, increased apoptosis in early B cell progenitors seems to underlie the defects in B cell development and may be partially due to de-repression of Bim [113], a protein known to regulate lymphocyte apoptosis [117, 118]. In addition, with mechanisms analogous to those described in ESCs, miR-17~92 appears to be required for the regulation of progenitor cell fate during tissue development. In the heart, miR-17~92 is transcriptionally regulated by BMP signals through conserved Smad binding sites on its promoter [119]. Both BMP4 and miR-17~92 null embryos display heart defects, which are exacerbated in compound mutants, indicating that the two genetically interact during heart development. Some of these defects have been linked to improper silencing of cardiac progenitor genes such as *Isl1* and *Tbx1*, both of which are direct targets of miR-17~92. These results demonstrate that like in ESCs, expression of miR-17~92 in the heart is important to ensure proper down-regulation of genes that promote the undifferentiated state. Although studied in less depth, miR-17~92 has also been implicated in the regulation of cell fate decisions in the lung where its overexpression promotes proliferation of epithelial progenitors and inhibits their differentiation, possibly by targeting *Rbl2* [120].

The role of miR-17~92 during embryonic development appears to be conserved. In humans, germline deletion of one copy of this cluster has been recently shown to be responsible for a developmental disorder known as Feingold Syndrome [121], whose core features affect the skeleton and are phenocopied in mice heterozygous for miR-17~92 [116]. Until recently, *N-Myc* was the only gene whose mutation had been shown to cause this disorder [121]. The discovery that hemizygous loss of miR-17~92 in humans causes Feingold Syndrome makes it the first example of a miRNA whose mutation is responsible for a developmental defect in humans, and exposes a *N-Myc*/miR-17~92 axis in the regulation of skeletal differentiation. However, mechanistic insight into the role of the cluster in this process is still lacking.

miR-290~295 and miR-302~367

Like miR-17~92, miR-290~295 is essential for mouse development, and in its absence three quarters of the animals die in utero between E11.5 and E18.5 [114]. At E8.5 some of these embryos are found outside of the yolk-sac, a phenotype that has also been documented for genes involved in early embryonic patterning like *Nodal* [122] and its receptors *ActRIIA* and *B* [123]. Mutants for *Foxa2*, a transcription factor that —like *Nodal* signaling [124]— controls the specification of the mesendoderm during gastrulation, also display a similar phenotype with incomplete penetrance [125, 126]. This suggests that the mislocalization of

the miR-290~295 mutant embryos in regard to its extraembryonic tissues may be a consequence of improper patterning during the differentiation of the germ layers.

Gata6 is a transcriptional regulator of the endodermal fate [127] and activates the expression of miR-302~367 in mouse embryos [128]. Although a loss-of-function model has not been published for this cluster, the miR-302 family has been shown to regulate mesendoderm specification in a variety of organisms, by directly controlling the expression of Nodal agonists [115, 129]. In human ESCs, miR-302 represses *lefty1* and *lefty2* mRNAs and its absence creates an imbalance in Nodal signaling that results in an expansion of neuroectodermal derivatives at the expense of the endodermal and mesodermal lineages [115]. Since miR-303~367 and miR-290~295 share most of their miRNA-seeds, it is conceivable that the two clusters contribute to early germ layer specification by modulating Nodal signals, and that deregulation of this pathway is behind some of the defects seen in the miR-290~295 mutants.

Surviving miR-290~295 null animals are phenotypically normal, but show a severe depletion of primordial germ cells (PGCs) at day 5 after birth. This depletion, which in females results in sterility, is caused by a failure of the PGCs to be incorporated into the hindgut and consequently to migrate to and colonize the gonads [114]. This phenotype is similar to the one described in zebrafishes that lack both maternal and zygotic Dicer. In zebrafish, mislocalization of PGCs is caused by deregulation of the *cxcr-sdf1* chemokine pathway, which has been attributed to the absence of miR-430, a miRNA that belongs to the miR-302 family [130]. In mammals the *cxcr-sdf1* pathway is also targeted by the miR-302~367 cluster [131], opening the possibility that, as proposed above, miR-302~367 and miR-290~295 cooperate in the regulation of PGC migration.

ESC-miRNAs in cancer

Given their roles in the control of differentiation, proliferation and survival, it is not surprising that members of the miR-290~295, miR-302~367, miR-17~92 and miR-106b~25 clusters are often associated with human malignancies.

Most notably, miR-17~92 —also known as oncomir-1— is overexpressed in a variety of human cancers including lung carcinomas, B-cell lymphomas and retinoblastomas [73, 132, 133], frequently through an amplification of its locus [134, 135]. The mechanisms by which miR-17~92 promotes tumor formation are now starting to be elucidated and appear to depend on the physiological context. In the hematopoietic lineage, transgenic overexpression of the cluster in a model of B-cell lymphoma cooperates with c-Myc to give rise to highly malignant tumors that can evade the triggering of apoptosis in response to abnormal proliferation [133]. Escape from apoptosis relies heavily on miR-19a and miR-19b whose oncogenic activity depends at least in part on their ability to repress the tumor suppressor Pten [136, 137]. This anti-apoptotic activity is reminiscent of the role of miR-17~92 during normal B-cell development, where it promotes cell survival during the pro- to pre-B cell transition [113].

Recent studies of retinoblastoma also suggest an oncogenic role for both miR-17~92 and miR-106b~25 in this context [73]. Human retinoblastomas almost always harbor a mutation in the *pRb* gene, which enables cells to proliferate. The loci of both miR-17~92 and miR-106b~25 are amplified in a subset of these tumors, as well as in a subset of lesions from a murine model of retinoblastoma in which both *pRb* and its family member *p107* are deleted [73, 138]. In addition, members of these clusters are amongst the most highly expressed miRNAs in human retinoblastomas, indicating that they may play a role in the progression of this disease. Indeed, overexpression of miR-17~92 in *pRb/p107* double knockout mice, results in highly proliferative tumors that readily invade the optic nerve. In

contrast to what was observed in the case of lymphomas, retinoblastomas that overexpress miR-17~92 do not evade the apoptotic response, but rather counteract compensatory responses to the deletion of the Rb genes, to maintain a highly proliferative status. Tumors that lack both pRb and p107 upregulate the expression of several CDKIs, including p21^{Cip1} and p57^{Kip2}, both of which are direct targets of miR-17 and miR-20a. Indeed, inhibition of miR-17/20 in retinoblastoma cell lines suppress their proliferation and result in an increase in the levels of p21^{Cip1}, suggesting that it is a critical target of miR-17~92 in the context of retinoblastomas [73].

The roles of miR-290~295 and miR-302~367 in tumor progression are not so well understood. In humans, both miR-302~367 and miR-371~373 (a cluster from the miR-290 family) have been linked to the development of germ cell tumors, which is in line with a putative role for members of these clusters in the regulation of PGC function during development [74, 139]. In addition, miR-373 has been implicated in tumor invasion and metastasis [140]. These phenotypes have been attributed to the regulation of Lats2 and CD44 respectively [74, 140].

Perspective

Our understanding of the mechanisms by which miRNAs regulate biological processes is still limited. With respect to miRNAs associated with human disease, this lack of knowledge represents a major limitation in our ability to develop adequate treatments.

One of the difficulties we face is the identification of relevant targets. Recent insight into miRNA-mediated repression indicate that most of miRNAs lead to mild effects in protein output [24, 25] and that the control they exert in biological processes might depend on minor changes in the expression of a combination of genes, rather than strong modulation of a single target. In addition, the ability of a microRNA to repress their targets depends on their relative abundance in the cell [141] and can be affected by a number of other variables, including 3' UTR length, abundance of other miRNAs targeting the same transcript, and the presence of RNA-interacting proteins that may modulate miRNA accessibility. Overexpression experiments, often used as a mean to understand miRNA function, have to be interpreted with caution as they can lead to the modulation of genes that are not true physiological targets. The recent development of techniques that allow the purification of Ago2/mRNA/miRNA complexes [41, 142, 143], provide an appealing way to identify relevant miRNA-mRNA interactions. In addition, miRNA knockout mice are essential to assess to which degree biological process depend on the presence of specific miRNA genes. Although the increasing availability of miRNA-knockout strains has facilitated the analysis of their functions *in vivo*, the existence of redundant gene families in the genome can mask some of their biological roles.

We are beginning to decipher the complex network of interactions through which ESC-miRNAs control development and drive disease. Undoubtedly we still have a long way to go, but it is safe to assume that as we apply more sophisticated experimental and computational strategies, new insights into the biology of these miRNAs as well as a more refined understanding of how they control cellular processes will emerge.

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References

1. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*. 1993; 75(5):843–54. [PubMed: 8252621]
2. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell*. 1993; 75(5):855–62. [PubMed: 8252622]
3. Lim LP, et al. Vertebrate microRNA genes. *Science*. 2003; 299(5612):1540. [PubMed: 12624257]
4. Farh KK, et al. The widespread impact of mammalian MicroRNAs on mRNA repression and evolution. *Science*. 2005; 310(5755):1817–21. [PubMed: 16308420]
5. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009; 136(2):215–33. [PubMed: 19167326]
6. Bartel DP, Chen CZ. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nature reviews Genetics*. 2004; 5(5):396–400.
7. Hornstein E, Shomron N. Canalization of development by microRNAs. *Nature genetics*. 2006; 38(Suppl):S20–4. [PubMed: 16736020]
8. Stark A, et al. Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3' UTR evolution. *Cell*. 2005; 123(6):1133–46. [PubMed: 16337999]
9. Inui M, Martello G, Piccolo S. MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol*. 11(4):252–63. [PubMed: 20216554]
10. Young RA. Control of the embryonic stem cell state. *Cell*. 2011; 144(6):940–54. [PubMed: 21414485]
11. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*. 2009; 10(2):126–39. [PubMed: 19165215]
12. Lee Y, et al. MicroRNA genes are transcribed by RNA polymerase II. *The EMBO journal*. 2004; 23(20):4051–60. [PubMed: 15372072]
13. Denli AM, et al. Processing of primary microRNAs by the Microprocessor complex. *Nature*. 2004; 432(7014):231–5. [PubMed: 15531879]
14. Gregory RI, et al. The Microprocessor complex mediates the genesis of microRNAs. *Nature*. 2004; 432(7014):235–40. [PubMed: 15531877]
15. Lee Y, et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature*. 2003; 425(6956):415–9. [PubMed: 14508493]
16. Ruby JG, Jan CH, Bartel DP. Intronic microRNA precursors that bypass Drosha processing. *Nature*. 2007; 448(7149):83–6. [PubMed: 17589500]
17. Okamura K, et al. The mirtron pathway generates microRNA-class regulatory RNAs in *Drosophila*. *Cell*. 2007; 130(1):89–100. [PubMed: 17599402]
18. Lund E, et al. Nuclear export of microRNA precursors. *Science*. 2004; 303(5654):95–8. [PubMed: 14631048]
19. Ketting RF, et al. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes & development*. 2001; 15(20):2654–9. [PubMed: 11641272]
20. Knight SW, Bass BL. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. *Science*. 2001; 293(5538):2269–71. [PubMed: 11486053]
21. Grimson A, et al. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Molecular cell*. 2007; 27(1):91–105. [PubMed: 17612493]
22. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120(1):15–20. [PubMed: 15652477]
23. Lewis BP, et al. Prediction of mammalian microRNA targets. *Cell*. 2003; 115(7):787–98. [PubMed: 14697198]
24. Selbach M, et al. Widespread changes in protein synthesis induced by microRNAs. *Nature*. 2008; 455(7209):58–63. [PubMed: 18668040]

25. Baek D, et al. The impact of microRNAs on protein output. *Nature*. 2008; 455(7209):64–71. [PubMed: 18668037]
26. Kanellopoulou C, et al. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes & development*. 2005; 19(4):489–501. [PubMed: 15713842]
27. Papaioannou MD, et al. Sertoli cell Dicer is essential for spermatogenesis in mice. *Developmental biology*. 2009; 326(1):250–9. [PubMed: 19071104]
28. De Pietri Tonelli D, et al. miRNAs are essential for survival and differentiation of newborn neurons but not for expansion of neural progenitors during early neurogenesis in the mouse embryonic neocortex. *Development*. 2008; 135(23):3911–21. [PubMed: 18997113]
29. Wang Y, et al. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nature genetics*. 2007; 39(3):380–5. [PubMed: 17259983]
30. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 144(5):646–74. [PubMed: 21376230]
31. Okamura K, Lai EC. Endogenous small interfering RNAs in animals. *Nature reviews. Molecular cell biology*. 2008; 9(9):673–8.
32. Bernstein E, et al. Dicer is essential for mouse development. *Nature genetics*. 2003; 35(3):215–7. [PubMed: 14528307]
33. Wienholds E, et al. The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nature genetics*. 2003; 35(3):217–8. [PubMed: 14528306]
34. Murchison EP, et al. Characterization of Dicer-deficient murine embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102(34):12135–40. [PubMed: 16099834]
35. Calabrese JM, et al. RNA sequence analysis defines Dicer's role in mouse embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104(46):18097–102. [PubMed: 17989215]
36. Houbaviy HB, Murray MF, Sharp PA. Embryonic stem cell-specific MicroRNAs. *Dev Cell*. 2003; 5(2):351–8. [PubMed: 12919684]
37. Suh MR, et al. Human embryonic stem cells express a unique set of microRNAs. *Developmental biology*. 2004; 270(2):488–98. [PubMed: 15183728]
38. Lipchina I, et al. Genome-wide identification of microRNA targets in human ES cells reveals a role for miR-302 in modulating BMP response. *Genes & development*. 25(20):2173–86. [PubMed: 22012620]
39. Houbaviy HB, et al. Characterization of a highly variable eutherian microRNA gene. *RNA*. 2005; 11(8):1245–57. [PubMed: 15987809]
40. Sinkkonen L, et al. MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. *Nat Struct Mol Biol*. 2008; 15(3):259–67. [PubMed: 18311153]
41. Chi SW, et al. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature*. 2009; 460(7254):479–86. [PubMed: 19536157]
42. Leung AK, et al. Genome-wide identification of Ago2 binding sites from mouse embryonic stem cells with and without mature microRNAs. *Nat Struct Mol Biol*. 18(2):237–44. [PubMed: 21258322]
43. Evans MJ, Kaufman MH. Establishment in culture of pluripotent cells from mouse embryos. *Nature*. 1981; 292(5819):154–6. [PubMed: 7242681]
44. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1981; 78(12):7634–8. [PubMed: 6950406]
45. Nichols J, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*. 1998; 95(3):379–91. [PubMed: 9814708]
46. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature genetics*. 2000; 24(4):372–6. [PubMed: 10742100]

47. Mitsui K, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*. 2003; 113(5):631–42. [PubMed: 12787504]
48. Chambers I, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*. 2003; 113(5):643–55. [PubMed: 12787505]
49. Avilion AA, et al. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes & development*. 2003; 17(1):126–40. [PubMed: 12514105]
50. Cole MF, et al. Tcf3 is an integral component of the core regulatory circuitry of embryonic stem cells. *Genes & development*. 2008; 22(6):746–55. [PubMed: 18347094]
51. Yu J, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007; 318(5858):1917–20. [PubMed: 18029452]
52. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006; 126(4):663–76. [PubMed: 16904174]
53. Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature*. 2007; 448(7151):313–7. [PubMed: 17554338]
54. Kim J, et al. An extended transcriptional network for pluripotency of embryonic stem cells. *Cell*. 2008; 132(6):1049–61. [PubMed: 18358816]
55. Marson A, et al. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell*. 2008; 134(3):521–33. [PubMed: 18692474]
56. Boyer LA, et al. Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell*. 2005; 122(6):947–56. [PubMed: 16153702]
57. Boyer LA, et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature*. 2006; 441(7091):349–53. [PubMed: 16625203]
58. O'Donnell KA, et al. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature*. 2005; 435(7043):839–43. [PubMed: 15944709]
59. Sridharan R, et al. Role of the murine reprogramming factors in the induction of pluripotency. *Cell*. 2009; 136(2):364–77. [PubMed: 19167336]
60. Nakagawa M, et al. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nature biotechnology*. 2008; 26(1):101–6.
61. Davis AC, et al. A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes & development*. 1993; 7(4):671–82. [PubMed: 8458579]
62. Schulte JH, et al. MYCN regulates oncogenic MicroRNAs in neuroblastoma. *International journal of cancer Journal international du cancer*. 2008; 122(3):699–704. [PubMed: 17943719]
63. Northcott PA, et al. The miR-17/92 polycistron is up-regulated in sonic hedgehog-driven medulloblastomas and induced by N-myc in sonic hedgehog-treated cerebellar neural precursors. *Cancer research*. 2009; 69(8):3249–55. [PubMed: 19351822]
64. Varlakhanova NV, et al. myc maintains embryonic stem cell pluripotency and self-renewal. *Differentiation; research in biological diversity*. 2010; 80(1):9–19.
65. Smith KN, Singh AM, Dalton S. Myc represses primitive endoderm differentiation in pluripotent stem cells. *Cell stem cell*. 2010; 7(3):343–54. [PubMed: 20804970]
66. Subramanyam D, et al. Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells. *Nature biotechnology*. 2011; 29(5):443–8.
67. Li Z, et al. Small RNA-mediated regulation of iPS cell generation. *The EMBO journal*. 2011; 30(5):823–34. [PubMed: 21285944]
68. Judson RL, et al. Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nature biotechnology*. 2009; 27(5):459–61.
69. Anokye-Danso F, et al. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell stem cell*. 2011; 8(4):376–88. [PubMed: 21474102]
70. Wang Y, et al. Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. *Nature genetics*. 2008; 40(12):1478–83. [PubMed: 18978791]
71. Hong H, et al. Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature*. 2009; 460(7259):1132–5. [PubMed: 19668191]

72. Ivanovska I, et al. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. *Molecular and cellular biology*. 2008; 28(7):2167–74. [PubMed: 18212054]
73. Conkrite K, et al. miR-17~92 cooperates with RB pathway mutations to promote retinoblastoma. *Genes & development*. 2011; 25(16):1734–45. [PubMed: 21816922]
74. Voorhoeve PM, et al. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell*. 2006; 124(6):1169–81. [PubMed: 16564011]
75. Dews M, et al. The myc-miR-17~92 axis blunts TGF{beta} signaling and production of multiple TGF{beta}-dependent antiangiogenic factors. *Cancer research*. 2010; 70(20):8233–46. [PubMed: 20940405]
76. Melton C, Judson RL, Blueloch R. Opposing microRNA families regulate self-renewal in mouse embryonic stem cells. *Nature*. 2010; 463(7281):621–6. [PubMed: 20054295]
77. Roush S, Slack FJ. The let-7 family of microRNAs. *Trends in cell biology*. 2008; 18(10):505–16. [PubMed: 18774294]
78. Landgraf P, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*. 2007; 129(7):1401–14. [PubMed: 17604727]
79. Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. *Science*. 2008; 320(5872):97–100. [PubMed: 18292307]
80. Newman MA, Thomson JM, Hammond SM. Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. *RNA*. 2008; 14(8):1539–49. [PubMed: 18566191]
81. Nam Y, et al. Molecular basis for interaction of let-7 microRNAs with Lin28. *Cell*. 2011; 147(5):1080–91. [PubMed: 22078496]
82. Rybak A, et al. A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. *Nature cell biology*. 2008; 10(8):987–93.
83. Heo I, et al. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Molecular cell*. 2008; 32(2):276–84. [PubMed: 18951094]
84. Lehrbach NJ, et al. LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in *Caenorhabditis elegans*. *Nature structural & molecular biology*. 2009; 16(10):1016–20.
85. Choi YJ, et al. miR-34 miRNAs provide a barrier for somatic cell reprogramming. *Nature cell biology*. 2011; 13(11):1353–60.
86. Stadtfeld M, Hochedlinger K. Induced pluripotency: history, mechanisms, and applications. *Genes & development*. 2010; 24(20):2239–63. [PubMed: 20952534]
87. Marion RM, et al. A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. *Nature*. 2009; 460(7259):1149–53. [PubMed: 19668189]
88. Utikal J, et al. Immortalization eliminates a roadblock during cellular reprogramming into iPS cells. *Nature*. 2009; 460(7259):1145–8. [PubMed: 19668190]
89. Lange C, Huttner WB, Calegari F. Cdk4/cyclinD1 overexpression in neural stem cells shortens G1, delays neurogenesis, and promotes the generation and expansion of basal progenitors. *Cell stem cell*. 2009; 5(3):320–31. [PubMed: 19733543]
90. Zetterberg A, Larsson O. Kinetic analysis of regulatory events in G1 leading to proliferation or quiescence of Swiss 3T3 cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1985; 82(16):5365–9. [PubMed: 3860868]
91. Harbour JW, Dean DC. The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes & development*. 2000; 14(19):2393–409. [PubMed: 11018009]
92. Harbour JW, et al. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell*. 1999; 98(6):859–69. [PubMed: 10499802]
93. Liu J, Levens D. Making myc. *Current topics in microbiology and immunology*. 2006; 302:1–32. [PubMed: 16620023]
94. Bartek J, Lukas J. Pathways governing G1/S transition and their response to DNA damage. *FEBS letters*. 2001; 490(3):117–22. [PubMed: 11223026]
95. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & development*. 1999; 13(12):1501–12. [PubMed: 10385618]

96. Stead E, et al. Pluripotent cell division cycles are driven by ectopic Cdk2, cyclin A/E and E2F activities. *Oncogene*. 2002; 21(54):8320–33. [PubMed: 12447695]
97. Savatier P, et al. Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. *Oncogene*. 1996; 12(2):309–22. [PubMed: 8570208]
98. Faast R, et al. Cdk6-cyclin D3 activity in murine ES cells is resistant to inhibition by p16(INK4a). *Oncogene*. 2004; 23(2):491–502. [PubMed: 14724578]
99. Sengupta S, et al. MicroRNA 92b controls the G1/S checkpoint gene p57 in human embryonic stem cells. *Stem cells*. 2009; 27(7):1524–8. [PubMed: 19544458]
100. Koledova Z, et al. Cell-cycle regulation in embryonic stem cells: centrosomal decisions on self-renewal. *Stem cells and development*. 2010; 19(11):1663–78. [PubMed: 20594031]
101. Koledova Z, et al. Cdk2 inhibition prolongs G1 phase progression in mouse embryonic stem cells. *Stem cells and development*. 2010; 19(2):181–94. [PubMed: 19737069]
102. Neganova I, et al. Expression and functional analysis of G1 to S regulatory components reveals an important role for CDK2 in cell cycle regulation in human embryonic stem cells. *Oncogene*. 2009; 28(1):20–30. [PubMed: 18806832]
103. Filipczyk AA, et al. Differentiation is coupled to changes in the cell cycle regulatory apparatus of human embryonic stem cells. *Stem cell research*. 2007; 1(1):45–60. [PubMed: 19383386]
104. Calegari F, et al. Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2005; 25(28):6533–8. [PubMed: 16014714]
105. Calegari F, Huttner WB. An inhibition of cyclin-dependent kinases that lengthens, but does not arrest, neuroepithelial cell cycle induces premature neurogenesis. *Journal of cell science*. 2003; 116(Pt 24):4947–55. [PubMed: 14625388]
106. Calo E, et al. Rb regulates fate choice and lineage commitment in vivo. *Nature*. 2010; 466(7310):1110–4. [PubMed: 20686481]
107. Porse BT, et al. E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis in vivo. *Cell*. 2001; 107(2):247–58. [PubMed: 11672531]
108. Stefani G, Slack FJ. Small non-coding RNAs in animal development. *Nature reviews Molecular cell biology*. 2008; 9(3):219–30.
109. Benetti R, et al. A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. *Nature structural & molecular biology*. 2008; 15(9):998.
110. Feldman N, et al. G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. *Nature cell biology*. 2006; 8(2):188–94.
111. Li JY, et al. Synergistic function of DNA methyltransferases Dnmt3a and Dnmt3b in the methylation of Oct4 and Nanog. *Molecular and cellular biology*. 2007; 27(24):8748–59. [PubMed: 17938196]
112. McCabe MT, Davis JN, Day ML. Regulation of DNA methyltransferase 1 by the pRb/E2F1 pathway. *Cancer research*. 2005; 65(9):3624–32. [PubMed: 15867357]
113. Ventura A, et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell*. 2008; 132(5):875–86. [PubMed: 18329372]
114. Medeiros LA, et al. Mir-290-295 deficiency in mice results in partially penetrant embryonic lethality and germ cell defects. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108(34):14163–8. [PubMed: 21844366]
115. Rosa A, Spagnoli FM, Brivanlou AH. The miR-430/427/302 family controls mesendodermal fate specification via species-specific target selection. *Developmental cell*. 2009; 16(4):517–27. [PubMed: 19386261]
116. de Pontual L, et al. Germline deletion of the miR-17 approximately 92 cluster causes skeletal and growth defects in humans. *Nature genetics*. 2011; 43(10):1026–30. [PubMed: 21892160]
117. Bouillet P, et al. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science*. 1999; 286(5445):1735–8. [PubMed: 10576740]

118. Egle A, et al. Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101(16):6164–9. [PubMed: 15079075]
119. Wang J, et al. Bmp signaling regulates myocardial differentiation from cardiac progenitors through a MicroRNA-mediated mechanism. *Developmental cell*. 2010; 19(6):903–12. [PubMed: 21145505]
120. Lu Y, et al. Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. *Developmental biology*. 2007; 310(2):442–53. [PubMed: 17765889]
121. Marcelis CL, et al. Genotype-phenotype correlations in MYCN-related Feingold syndrome. *Human mutation*. 2008; 29(9):1125–32. [PubMed: 18470948]
122. Lowe LA, Yamada S, Kuehn MR. Genetic dissection of nodal function in patterning the mouse embryo. *Development*. 2001; 128(10):1831–43. [PubMed: 11311163]
123. Song J, et al. The type II activin receptors are essential for egg cylinder growth, gastrulation, and rostral head development in mice. *Developmental biology*. 1999; 213(1):157–69. [PubMed: 10452853]
124. Fei T, et al. Smad2 mediates Activin/Nodal signaling in mesendoderm differentiation of mouse embryonic stem cells. *Cell research*. 2010; 20(12):1306–18. [PubMed: 21079647]
125. Dufort D, et al. The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. *Development*. 1998; 125(16):3015–25. [PubMed: 9671576]
126. Bartscher I, Lickert H. Foxa2 regulates polarity and epithelialization in the endoderm germ layer of the mouse embryo. *Development*. 2009; 136(6):1029–38. [PubMed: 19234065]
127. Morrissey EE, et al. GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. *Genes & development*. 1998; 12(22):3579–90. [PubMed: 9832509]
128. Tian Y, et al. Regulation of lung endoderm progenitor cell behavior by miR302/367. *Development*. 2011; 138(7):1235–45. [PubMed: 21350014]
129. Choi WY, Giraldez AJ, Schier AF. Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. *Science*. 2007; 318(5848):271–4. [PubMed: 17761850]
130. Staton AA, Knaut H, Giraldez AJ. miRNA regulation of Sdf1 chemokine signaling provides genetic robustness to germ cell migration. *Nature genetics*. 2011; 43(3):204–11. [PubMed: 21258340]
131. Fareh M, et al. The miR 302–367 cluster drastically affects self-renewal and infiltration properties of glioma-initiating cells through CXCR4 repression and consequent disruption of the SHH-GLI-NANOG network. *Cell death and differentiation*. 2012; 19(2):232–44. [PubMed: 21720384]
132. Hayashita Y, et al. A polycistronic microRNA cluster, miR-17–92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer research*. 2005; 65(21):9628–32. [PubMed: 16266980]
133. He L, et al. A microRNA polycistron as a potential human oncogene. *Nature*. 2005; 435(7043):828–33. [PubMed: 15944707]
134. Ota A, et al. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer research*. 2004; 64(9):3087–95. [PubMed: 15126345]
135. Tagawa H, Seto M. A microRNA cluster as a target of genomic amplification in malignant lymphoma. *Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, UK*. 2005; 19(11):2013–6.
136. Mu P, et al. Genetic dissection of the miR-17~92 cluster of microRNAs in Myc-induced B-cell lymphomas. *Genes & development*. 2009; 23(24):2806–11. [PubMed: 20008931]
137. Olive V, et al. miR-19 is a key oncogenic component of mir-17–92. *Genes & development*. 2009; 23(24):2839–49. [PubMed: 20008935]
138. Chen D, et al. Cell-specific effects of RB or RB/p107 loss on retinal development implicate an intrinsically death-resistant cell-of-origin in retinoblastoma. *Cancer cell*. 2004; 5(6):539–51. [PubMed: 15193257]

139. Palmer RD, et al. Malignant germ cell tumors display common microRNA profiles resulting in global changes in expression of messenger RNA targets. *Cancer research*. 2010; 70(7):2911–23. [PubMed: 20332240]
140. Huang Q, et al. The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. *Nature cell biology*. 2008; 10(2):202–10.
141. Mukherji S, et al. MicroRNAs can generate thresholds in target gene expression. *Nature genetics*. 2011; 43(9):854–9. [PubMed: 21857679]
142. Hafner M, et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell*. 2010; 141(1):129–41. [PubMed: 20371350]
143. Lal A, et al. Capture of microRNA-bound mRNAs identifies the tumor suppressor miR-34a as a regulator of growth factor signaling. *PLoS genetics*. 2011; 7(11):e1002363. [PubMed: 22102825]

Figure 1. Cell Cycle control by ESC-miRNAs

Progression through G1 requires the formation of an active CyclinE/CDK2 complex in response to mitogenic signals. In embryonic stem cells, miRNAs from the four ESC-clusters contribute to a short G1 phase by repressing several CDK inhibitors as well as members of the Rb family. Promoters are depicted as orange boxes, proteins as blue ovals and miRNAs as purple boxes.

Figure 2. Integration of the four ESC-clusters in the pluripotency circuitry

Members of the four microRNA clusters control a variety of cellular processes involved in ESC function, which include signaling events, cell cycle and chromatin modifications. As before, promoters are depicted as orange boxes, proteins as blue ovals and miRNAs as purple boxes.

Table 1**The four ESC-miRNA clusters**

The sequences of the mature miRNAs belonging to the four “ESC miRNA clusters” are shown. Seed sequences are highlighted in color according to sequence similarity.

Gene	Mature miRNAs	Mature Sequence	
miR-290~295			
	miR-290	AAAGUGCCGCCUAGUUUUAGCCC	Embryonic lethal with incomplete penetrance. Defects in early embryonic patterning and surviving females
	miR-291a	AAAGUGCUUCCACUUUGUGUGC	
	miR-292	AAAGUGCCGCCAGGUUUUGAGUGU	
	miR-291b	AAAGUGCAUCCAUUUUGUUUGU	
	miR-293	AGUGCCGCAGAGUUUGUAGUGU	
	miR-294	AAAGUGCUUCCCUUUUGUGUGU	
	miR-295	AAAGUGCCGCCUAGUUUUAGCCC	
miR-17~92			
	miR-17	CAAAGUGCUUACAGUGCAGGUAG	KO animals die perinatally displaying lung hypoplasia, cardiac defects, block in B cell differentiation and skeletal development
	miR-18a	UAAGGUGCAUCUAGUGCAGAUAG	
	miR-19a	UGUGCAAAUCUAUGCAAAACUGA	
	miR-20a	UAAAGUGCUUAUAGUGCAGGUAG	
	miR-19b-1	UGUGCAAAUCCAUGCAAAACUGA	
	miR-92a-1	UAUUGCACUUGUCCCGGCCUG	
miR-302~367			
	miR-302b	UAAGUGCUUCCAUGUUUUAGUAG	Normal. Cooperates with miR double knockouts die before E15.5 with agravated phenotypes
	miR-302c	AAGUGCUUCCAUGUUUCAGUGG	
	miR-302a	UAAGUGCUUCCAUGUUUUGGUGA	
	miR-302d	UAAGUGCUUCCAUGUUUGAGUGU	
	miR-367	AAUUGCACUUUAGCAAUGGUGA	
miR-106b~25			
	miR-106b	UAAAGUGCUGACAGUGCAGAU	
	miR-93	CAAAGUGCUGUUCGUGCAGGUAG	
	miR-25	CAUUGCACUUGUCUCGGUCUGA	